Hydrolysis and transgalactosylation catalysed by β-galactosidase from brush border membrane vesicles isolated from pig small intestine: A study using lactulose and its mixtures with lactose or galactose as substrates

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ABSTRACT

Enzymatic transgalactosylation, in different concentrated carbohydrate solutions, was investigated using brush border membrane vesicles (BBMV) from the pig small intestine. When lactulose was incubated with BBMV, the hydrolytic activity of the enzyme towards the disaccharide was observed to be very low compared to that towards the lactose, but the linkage specificity β–(1→3), previously observed in lactose solutions, was not significantly affected. As in the case of lactose, lactulose transgalactosylation by BBMV synthesizes the corresponding 3′-galactosyl derivative (β-Gal-(1→3)-β-Gal-(1→4)-β-Fru). Fructose released during lactulose hydrolysis was found to be good acceptor for the transgalactosylation reaction, giving rise to the synthesis of the disaccharide β-Gal-(1→5)-Fru. When incubating an 80/20 mixture of lactulose/galactose, the presence of galactose did not affect the qualitative composition of the transglycosylated substrate but enhanced the synthesis of β-Gal-(1→5)-Fru and decreased the synthesis of β-(1→3) glycosidic bonds. The marked tendency for synthesizing this linkage indicates that under hydrolytic conditions, β-Gal-(1→3)-Gal- and β-Gal-(1→5)-Fru glycosidic bonds would be preferentially digested.

Keywords: lactulose, lactose, digestibility, transgalactosylation, pig small intestinal BBMV.

1. Introduction

Digestion of carbohydrates requires a series of enzymatic reactions to break them down into monosaccharides. This is achieved by the combined action of pancreatic enzymes and enzymes at the brush border of the small intestine (Hernandez-Hernandez et al., 2019). α−Amylase catalyzes the first step in the digestion of starch, giving rise to the formation of maltose, isomaltose, maltotriose, and α-dextrins. These compounds as well as dietary disaccharides (sucrose and lactose) are digested by the action of glycosidases (sucrase-isomaltase, lactase, maltase-glucoamylase, and trehalase) located on the brush border membrane vesicles (BBMV) (Holmes, 1971). Non-digestible carbohydrates, called dietary fibers, can be insoluble or soluble (Lin et al., 2019; Mussatto & Mancilha, 2007). Insoluble fibers pass through the digestive tract relatively unaltered increasing stool bulk, helping it to pass faster through the intestine whereas soluble fiber is fermented in the colon. When soluble fibers are selectively fermented by beneficial bacteria conferring a health benefit they are classified as prebiotics (Carlson, Erickson, Lloyd & Slavin, 2018; Holscher, 2017).

The extent of digestion before reaching the colon is important because it determines not only the glycemic response but also the proportion of dietary fiber available to the colon for fermentation. It is well-known that prebiotics exert a remarkable influence on human health, which makes them attractive agents to improve the quality of human life by preventing different pathologies (Davani-Davari et al., 2019).

Detailed knowledge of the partial digestion pathways in the small intestine, and fermentation mechanisms of such oligosaccharides would facilitate the enhancement of

prebiotic functionality and, to some extent, the design of carbohydrates targeted at particular species of probiotic microorganisms.

Although there are a considerable number of oligosaccharides with a wide diversity of structures proposed as potential prebiotics, the only four that are well supported by solid data from human trials are the fructans (inulin and fructo-oligosaccharides, FOS), galacto-oligosaccharides (GOS), and the synthetic disaccharide, lactulose (O' Bryan et al., 2013; Roberfroid, 2008).

GOS are synthetized from lactose by transgalactosylation catalyzed by microbial β-galactosidases and consist of β-(1 → 2, 3, 4, or 6) linked galactose units with terminal glucose (Torres, Gonçalves, Teixeira & Rodrigues, 2010). GOS belong to the group of the short chain carbohydrates that are slowly absorbed or indigestible and not absorbed in the small intestine (Ferreira-Lazarte et al., 2019). A large number of microorganisms having different regiochemical selectivity have been assessed as potential sources of β-galactosidases (Yin, Dijkhuizen, van Leeuwen, 2018; Gonzalez-Delgado, López-Muñoz, Morales & Segura, 2016; Gänzle, 2012; Panesar, Kumari & Panesar, 2010) and the products obtained consist of complex mixtures of mono- di- and oligosaccharides. Besides the differences in purity of GOS mixtures, linkages specificity varies with the origin of the enzyme so that, the structures. Depending on their chemical structures, GOS products will vary in terms of resistance to enzymatic hydrolysis in the small intestine, as well as other physiological effects (Hernandez-Hernandez et al., 2012).

Lactulose (4-*O*-β-D-galactopyranosyl–D-fructose) is a synthetic sugar, which does not occur naturally and is produced industrially from lactose by isomerization in basic media using different types of catalyst (Olano & Corzo, 2009). It is also produced

during heat treatment of milk and therefore, it may be present in considerable amounts in heat-processed dairy products. Lactulose is mainly used in medicine for the treatment of portal systemic encephalopathy and chronic constipation and it is one of the most widely used prebiotic in the pharmaceutical industry (Zucker & Redulla, 2019; Villamiel, Montilla, Olano & Corzo, 2014). Lactulose may be used to produce lactulose-derived galacto-oligosaccharides (OsLu) by enzyme-mediated transgalactosylation (Sabater et al., 2019; Young et al., 2019; Yin, Dijkhuizen & van Leeuwen, 2018; Cardelle-Cobas et al., 2008; 2016).

A number of studies have shown the mechanisms of transgalactosylation of lactose and lactulose for synthesis of GOS catalyzed by β-galactosidases of different microbial origins (Yin et al., 2018; Díez-Municio et al., 2014; Gänzle, 2012; Panesar, Kumari & Panesar, 2010; Torres et al., 2010); however, few data are available on the enzymatic transgalactosylation catalyzed by the small intestinal BBMV (Julio-Gonzalez et al., 2019). Since hydrolysis and transglycosylation occurs simultaneously at the same active site of the glycosyl hydrolases (Abdul Manas et al., 2018), to address our understanding of the mechanism of hydrolysis of mammalian BBMV enzymes, the preferential synthesis of 3'galactosyl-lactose by forming a β-(1→3)-glycosidic bonds was recently demonstrated for the first time by using pig small intestinal BBMV and lactose (Julio-González et al., 2019). Kinetic analysis of the synthesized carbohydrates showed that the β–(1→3) transfer product was the main one formed rapidly and continuously. The research was performed with lactose as the only substrate; however, foods are a complex mixture of compounds which can affect the enzymatic reactions catalyzed by small intestinal disaccharidases.

The study of the mechanisms of transgalactosylation of mixtures of dietary carbohydrates by BBMV may contribute to the knowledge of the digestion mechanisms in the small intestine, and provide further insight into the structures of oligosaccharides, required for the effective design of new prebiotics with improved or complementary properties.

This study looked at how the presence of other sugars such as lactulose (isomer of lactose) and mixtures of lactulose/lactose and lactulose/galactose affects the hydrolysis and the transgalactosylation by the pig small intestinal BBMV and compares the findings with those previously obtained for lactose.

2. Materials and methods

2.1. Chemicals

In this work, all used chemicals and reagents were of analytical grade. Galactose, glucose, fructose, lactose, allolactose, lactulose, raffinose and phenyl-β-glucoside were acquired from Sigma-Aldrich (St Louis, MO, USA). Galactobiose, Gal (1→4) Gal, was purchased from Carbosynth (Berkshire, UK).

2.2 Preparation of pig small intestinal brush border membrane vesicles (BBMV)

BBMV were obtained following the methodology previously proposed (Kesslet et al., 1978; Tanabe et al., 2015). Briefly, three pig small intestines, from the duodenum to the ileum, were obtained from a local slaughterhouse (Coca, Spain). Immediately after sacrifice, the samples were kept at 4 °C and transferred to the laboratory in less than 2 hours. The small intestines were rinsed with cold phosphate buffered saline solution (PBS) (pH 7.3–Oxoid; Basingstoke, UK), then slit open and scrapped with a glass slide. The mucosal scrapped was suspended (1:1, w/v) in 50 mM mannitol

dissolved in PBS at 4 °C, homogenized during 10 min using a Ultra-Turrax® (IKA T18 Basic), adjusted with $CaCl_2$ to a final concentration of 10 mM and centrifuged at 3,000 ×g during 30 min. The supernatant was centrifuged at 27,000 ×g during 40 min and the resulting pellet, containing the BBMV, was re-suspended in buffer maleate (50 mM) pH 6.0 containing $CaCl_2$ (2 mM) and sodium azide (0.02%). Samples were lyophilized and kept at −80 °C.

2.3. Synthesis of oligosaccharides derived from lactulose and its binary mixtures with lactose or galactose

Oligosaccharides were synthesized following the method previously described by Julio-Gonzalez et al (2019) with some modifications. Enzymatic reactions were carried out in triplicate using lactulose (25 mg/mL) or mixtures of lactulose/lactose (50:50) or lactulose/galactose (80:20), in PBS (pH 7.3) and lyophilized BBMV (180 mg/mL), at 37 ºC in an orbital shaker at 900 rpm. Samples aliquots were withdrawn at specific time intervals (0, 2 and 4 h) and immediately immersed in boiling water for 5 minutes to inactivate the enzyme, and then stored at -20 º C for subsequent analysis.

β-galactosidase activity is expressed in units per gram (U/g) where 1 unit is defined as the amount of enzyme hydrolysing 1 μmol of lactose per minute under the assayed conditions. β-galactosidase activity in BBMV was 14.87 U/g.

2.4. Purification and isolation of trisaccharide derived from lactulose synthesized by pig small intestinal BBMV

2.4.1 Purification using activated charcoal treatment

In order to remove mono- and disaccharides, oligosaccharide mixtures, obtained after 6 h of reaction, were purified using activated charcoal (powder, Sigma-Aldrich, St.

Louis, USA) following the methodology proposed by Julio-González, Ruiz, Corzo & Olano, (2018). Briefly, oligosaccharide mixture (500 mg) and activated charcoal (3 g) were added to 100 mL of ethanol (8%, v/v). The mixture was stirred for 30 min at 25ºC and then filtered through Whatman No.1 paper (Whatman International Ltd., Maidstone, UK) under vacuum.

Desorption of trisaccharides from the activated charcoal was carried out with 100 mL ethanol (50%, v/v). The mixture was stirred for 30 min and filtered as previously described and an aliquot was taken for further analysis.

2.4.2. Isolation by HILIC-RID

The trisaccharides desorbed from activated charcoal were isolated by Hidrophilic Interaction Liquid Chromatography coupled to a refractive index detector (HILIC-RID) using a semi-preparative ZORBAX NH_2 column (PrepHT cartridge 250 x 21.2 mm, 7 μm particle size) (Agilent,Technologies, Madrid, Spain). 2 mL of reaction mixtures repeatedly injected and eluted with acetonitrile:water (70:30, v/v) at a 21 mL/min flow rate. The fractions containing the main trisaccharide were collected using an Agilent Technologies 1260 Infinity preparative fraction collector (Boeblinger, Germany), pooled, evaporated in a rotatory evaporator R-200 (Büchi, Flawil, Switzerland) and lyophilized for subsequent GC-MS and NMR characterization.

2.5. Quantification of carbohydrates by Gas Chromatography-Flame Ionization Detector (GC –FID)

Carbohydrates present in all enzymatic reaction mixtures were analyzed by GC-FID as trimethylsilyl oximes (TMSO) which were prepared following the method of Brobst & Lott (1966). First, oximes were formed by addition of 300 μL of hydroxylamine

chloride (2.5% w/v) in pyridine to dried samples and heating the mixture at 70ºC for 30 min. The resulting oximes were then silylated with hexamethyldisilazane (300 µL) and trifluoroacetic acid (30 µL) at 50 ºC for 30 min. After reaction, samples were centrifuged at 10,000 rpm for 2 min at room temperature, and 1 µL of supernatants was injected into the GC injection port or stored at 4ºC prior to analysis.

Analysis of carbohydrates as TMSO derivatives was carried out in a Agilent Technologies gas chromatograph (Mod 7890A) equipped with a flame ionization detector (FID) using a fused silica capillary column DB-5HT, crosslinked phase (5%-phenyl-methylpolysiloxane; 30m x 0.25mm x 0.10µm) (Agilent J&W Scientific, Folsom, CA, USA). The oven temperature was initially set at 150 ºC and then programmed to 380 ºC at 3 ºC/min. The injector and detector temperatures were set at 280 and 385 ˚C, respectively. Injections were carried out in split mode (1:20) using nitrogen at 1 mL/min as carrier gas. Data acquisition and integration were performed using Agilent ChemStation software. Quantification was performed by internal standard method using phenyl-β–glucoside (0.5 mg/mL). To calculate response factors, solutions containing glucose, fructose, galactose, lactulose and raffinose (used as standard of trisaccharides) were prepared over the expected concentration range in the samples. Carbohydrates concentration was expressed as mg/100mg of substrate. All analyses were carried out in duplicate and data were expressed as mean ± standard deviation (SD).

2.6. GC-MS characterisation and identification of transglycosylated disaccharides

TMSO of disaccharides present in reaction mixtures were analysed in a Hewlett-Packard 6890 gas chromatograph coupled to a 5973 quadrupole mass detector (Agilent, Palo Alto, CA, USA). Separation of carbohydrates was carried out in a fused silica

capillary column DB-5HT (5%-phenyl-methylpolysiloxane; 30m x 0.25mm x 0.10µm) (Agilent). The oven temperature was initially set at 150 ºC then programmed to 300 ºC at 3 ºC/min and kept for 10 min. Injector temperature was 300 ºC. Injections were carried out in split mode (1:20) using helium as carrier gas (0.8 mL/min). Mass spectrometer was operated in electronic impact (EI) mode at 70 eV with a scanning range comprised between 35 and 700 m/z. Interface and source temperature were 280 ºC and 230 ºC, respectively. Data acquisition was obtained using a HPChem Station software (Hewlett-Packard, Palo Alto, CA, USA).

The identification of TMSO derivatives of disaccharides was performed by comparison of their mass spectra with those standard compounds derivatised and previously reported (Hernández-Hernández et al., 2011; Sanz, Sanz & Martínez-Castro, 2002).

2.7. Structural characterization by NMR of trisaccharide derived from transgalactosylation of lactulose

Structure elucidation of trisaccharide was accomplished by Nuclear Magnetic Resonance spectroscopy (NMR). NMR spectra were recorded at 298 K, using D_2O as solvent, on an Agilent SYSTEM 500 NMR spectrometer (^1H 500 MHz, ^{13}C 125 MHz) equipped with a 5-mm HCN cold probe. Chemical shifts of ^1H (δ_H) and ^{13}C (δ_C) in parts per million were determined relative to internal standards of sodium [2, 2, 3, 3-$^2H_4$]-3-(trimethylsilyl)-propanoate in D_2O (δ_H 0.00) and 1,4-dioxane (δ_C 67.40) in D_2O, respectively. One-dimensional (1D) NMR experiments (^1H, and ^{13}C) were performed using standard pulse sequences. Two-dimensional (2D) [^1H, ^1H] NMR experiments [gradient correlation spectroscopy (gCOSY), total correlation spectroscopy (TOCSY), and rotating-frame Overhauser effect spectroscopy (ROESY)] were carried out with the

following parameters: delay time of 1 s, spectral width of 2962 Hz in both dimensions, 2048 complex points in t2, 16 transients (32 for ROESY) for each of 200 time increments, and linear prediction to 512. The data were zero-filled to 2048 × 2048 real points. A mixing time of 200 ms was used for ROESY experiment. 2D [$^{1}H-^{13}C$] NMR experiments [gradient heteronuclear single-quantum coherence (gHSQC) and gradient heteronuclear multiple-bond correlation (gHMBC)] used the same ^{1}H spectral window, a ^{13}C spectral window of 16336.5 Hz, 1 s of relaxation delay, 1024 data points, and 128 time increments, with a linear prediction to 256. The data were zero-filled to 2048 × 2048 real points. Typical numbers of transients per increment were 16 and 64, respectively (Julio-Gonzalez et al., 2018; Yin et al., 2018).

3. Results and discussion

3.1. Transgalactosylation by pig BBMV using lactulose as substrate

Figure 1 (a) shows the GC-FID profile obtained from lactulose transgalactosylation catalyzed by BBMV. It should be noted that reducing carbohydrates gave rise to two peaks corresponding to the TMSO of syn (*E*) and anti (*Z*) isomers and also named as 1 and 2 in the case of ketoses, after derivatization. The chromatograms show a weak β-galactosidase activity of BBMV towards lactulose in line with the prebiotic character of this disaccharide; in consequence, small amounts of transgalactosylated oligosaccharides DP2 and 3 were observed. In the disaccharide fraction (Figure 1a_1), apart from lactulose (peak 4), two new compounds were detected (peaks 6 and 7). These disaccharides were identified by GC-MS as β-Gal-(1→4)-Gal (1,4-galactobiose) (peaks number 6) by comparison of their GC-MS profile with the corresponding commercial standard, and β-Gal-(1→5)-Fru 1 and 2 (peaks number 7) by comparison with the data previously reported by Hernández-Hernández et al. (2011) and

the presence of the ion *m/z* 539 which is characteristic from (1→5) linkages (Sanz, Sanz & Martínez-Castro,. 2002).

Trisaccharide fraction (Figure 1a$_2$) was constituted by a main compound (peaks 11) which was isolated by HILIC-RID and characterized by NMR by the combined use of 1D and 2D [^{1}H, ^{1}H] and [^{1}H, ^{13}C] NMR experiments (gCOSY, TOCSY, ROESY, multiplicity-edited gHSQC and gHMBC). ^{1}H and ^{13}C NMR chemical shifts observed are summarized in Table 1. Full set of spectra are available in the Supporting Information (Figures S1–S7).

The 1D ^{1}H NMR spectrum of the trisaccharide fraction (Figure 2) showed three sets of two doublets in the anomeric region (δ4.610, J (H1, H2) =7.8 Hz and δ4.615, J (H1, H2) =7.8 Hz), (δ4.610, J (H1, H2) =7.8 Hz and δ4.510, J (H1, H2) ≈8 Hz), and (δ4.610, J (H1, H2) =7.8 Hz and δ4.49,J (H1, H2) ≈8 Hz) in a 67:23:10 ratio. In addition, the 1D ^{13}C NMR spectrum showed signals corresponding to three sets of 18 carbons, in the same ratio, each of them including three anomeric carbons [(δ104.98, δ101.10, and δ98.74 for the first anomeric form), (δ104.98, δ103.16.10, and δ103.01 for the second anomeric form), and (δ104.98, δ103.59, and δ105.60 for the third anomeric form)]. A multiplicity-edited gHSQC spectrum was used to link the carbon signals to the corresponding proton resonances. Each set showed three anomeric carbons, one of them a quaternary carbon, eleven CH and four methylene carbons. 2D COSY and TOCSY experiments and the coupling constant values revealed ^{1}H signals consistent with the structure of a trisaccharide with G1 and G2 units being β-D-galactopyranosyl and the reducing terminal G3 unit a fructose with β-pyranose (67%), β-furanose (23%) and α-furanose (10%) forms. Identification of the different tautomeric forms was performed on the basis of the ROESY correlations and ^{13}C chemical shifts. The position of glycosidic linkages was analyzed as follows: gHMBC showed, for the three forms,

correlations between the β-Gal-1-C1 anomeric carbon and β-Gal-2-H3 proton and between the β-Gal-2-H1 anomeric proton and the Fru-C4 carbon. These results confirmed the structure as β-D-galactopyranosyl-(1→3)- β-D-galactopyranosyl-(1→ 4)-β-D-fructose (Figure 3), in accord with other data from Yin, Dijkhuizen & van Leeuwen (2018) who used a microbial β-galactosidase. However, according to our knowledge, this manuscript reports the first NMR full assignment of all detectable tautomeric forms (β–pyranoside, β-furanoside and α-furanoside) present in an aqueous solution.

The time course of transgalactosylation of lactulose by BBMV is shown in Figure 4. Lactulose content decreased much slower than previously reported for lactose (Julio-Gonzalez, et al. 2019) so more than 70 % of initial lactulose remained unaltered after 4 h of reaction (Figure 4a). As consequence of the hydrolysis, fructose and galactose were released but their content was lower than that corresponding to the loss of lactulose. Moreover, galactose content was found to be lower than fructose which occurs only if a transgalactosylation reaction has taken place. Synthesis yield of β-Gal-(1→4)-Gal, β-Gal-(1→5)-Fru and 3'-galactosyl-lactulose increased with reaction time (Figure 4b). The formation of β-(1→3) glycoside was favorable for the synthesis of GOS by BBMV from lactose (Julio-González et al., 2019); however, fructose resulted to be the most favorable acceptor of galactose giving rise to the synthesis of β-Gal-(1→5)-Fru, the main product formed during transgalactosylation of lactulose. These results seem to indicate that lactulose acts not only as the glycosyl donor but also as acceptor so that fructose does not leave the acceptor binding site but changes position and the lactulose isomer β-Gal-(1→5)-Fru is formed. Lactulose and galactose also react with the glycosyl-enzyme intermediate to form 3'-galactosyl lactulose and β-Gal-(1→4)-Gal, respectively, although the latter was synthesized at a much lesser extent. β-

Gal-(1→5)-Fru was previously identified in galactose and fructose enzymatic reaction mixtures incubated with immobilized β-galactosidases from *Aspergillus oryzae* and *Escherichia coli (*Ajisaka, Fujimoto & Nishida, 1987), showing the capacity of fructose to act as a galactose acceptor.

3.2. Transgalactosylation by pig BBMV using a binary mixture of lactulose/galactose as substrate

When a mixture of lactulose/galactose 80/20 was used as a single substrate, the hydrolytic activity of the enzyme towards lactulose was not affected and the transgalactosylation products originated were the same that those found during transgalactosylation using lactulose as a single substrate **(**Figure 5). Disaccharide fraction was constituted by unreacted lactulose, β-Gal-(1→4)-Gal and β-Gal-(1→5)-Fru, and 3'-galactosyl-lactulose was the only trisaccharide yielded. However, the formation of β-Gal-(1→5)-Fru increased almost twice the content achieved during the transgalactosylation of single lactulose, whilst the trisaccharide 3'-galactosyl-lactulose decreased about twice (Figure 5b). Significant changes on β-Gal-(1→4)-Gal synthesis were not observed. This finding not only confirms that fructose is the more preferred acceptor substrate of the galactose moiety from the galactosyl–enzyme complex but also indicates that the presence of large amounts of galactose in the medium interfere with the synthesis of 3′-galactosyl-lactulose.

3.3. Transgalactosylation by pig BBMV using a binary mixture of lactulose/lactose as substrate

In a previous work, Julio-Gonzalez et al. (2019) studied the transgalactosylation mechanism of β-galactosidase from pig small intestinal BBMV to synthesize GOS using

lactose as the only substrate. Therefore, in order to study the specificity of β-galactosidase from BBMV towards lactose and lactulose, a mixture containing equimolecular amounts of both carbohydrates was transglycosylated. Figure 1 (b) shows the corresponding GC-FID chromatograms of the products found in these reaction mixtures. In this case, the hydrolysis of lactose and lactulose released fructose (peaks 1), galactose (peaks 2) and glucose (peaks 3). In the disaccharide region (Figure 1 b_1) apart from lactulose (peaks 4) and lactose (peaks 5), a series of compounds (peaks numbered from 6 to 10) were detected. These disaccharides were identified by GC-MS as β-Gal-(1→4)-Gal *E* (peak 6; 1,4-galactobiose); β-Gal-(1→5)-Fru 1 and β-Gal-(1 → 3)-Glc *E* (peaks 7 and 8, respectively), β-Gal-(1 →2)-Glc *E* (peak 9), β-Gal-(1→4)-Gal *Z* and β-Gal-(1 → 3)-Glc *Z* (peaks 6 and 8, respectively), β-Gal-(1→5)-Fru 2 and β-Gal-(1 → 2)-Glc *Z (*peaks 7 and 9, respectively) and β-Gal-(1→6)-Glc (allolactose; peaks 10) by comparison of their profiles with those commercial standards as well as those previously identified by GC-MS in transgalactosylation reaction mixtures of lactose by BBMV (Julio-González et al., 2019) and transglycosylated mixtures of lactulose by β−galactosidases from *Kluyveromyces lactis* (Lactozym 6500 L), *Aspergillus aculeatus* (Pectinex Ultra) and *Aspergillus oryzae* (Hernández-Hernández et al., 2011).

In the trisaccharide fraction (Figure $1b_2$) several peaks were detected which were identified as β-Gal-(1-3)-β-Gal-(1-4)-β-Fru 1 (peak 11; identified as 3'-galactosyl-lactulose); β-Gal-(1-3)-β-Gal-(1-4)-β-Fru 2 and β-Gal-(1-3)-β-Gal-(1-4)-β-Glc (3'-galactosyl-lactose) (peaks 11 and 13, respectively); and the mixture of two trisaccharides, β-Gal-(1→4)-β-Glc-(1↔1)-β-Gal and β-Gal-(1→3)-Glc-(2→1)-β-Gal (peak 12). Also, a sequence of very minor and unknown carbohydrates was detected (marked with asterisk, Figure $1b_2$).

Figure 6 shows the content of lactose, lactulose and released monosaccharides as well as di- and trisaccharides formed during hydrolysis and transgalactosylation of the equimolecular mixture of lactulose and lactose by pig small intestinal BBMV. In general, released monosaccharides and formed transgalactosylated products increased during time course of reaction. As expected, the hydrolysis of lactose was much faster than that of lactulose and the amounts of fructose, glucose and galactose present in samples were lower compared to the lactose and lactulose decrease, which is indicative of transglycosylation reaction. A considerable decrease of lactose (68%) was observed after the first 2h of reaction while lactulose remained almost unaltered. About 90% of lactose disappeared and 84% of lactulose remained unaltered after 4h of reaction.

As a result of transgalactosylation of galactose to fructose or galactose, β-Gal-(1 → 5)-Fru and β-Gal-(1 →4)–Gal (1,4-galactobiose) were also quantified at levels of 22.7 and 4.3% of the total disaccharide fraction (excluding lactulose and lactose), respectively. This is one of the main differences found with regard to transgalactosylated mixtures of lactose with β-galactosidase from pig BBMV because in this case only disaccharides β-galactosyl-glucoses linked by (1→2), (1→3) or (1→6) (allolactose) were identified (Julio- González et al., 2019).

Regarding trisaccharides formation, 3'-galactosyl-lactose was formed in greater amount (6% of total carbohydrates), followed by 3'-galactosyl-lactulose (2% of total carbohydrates) and a mixture of two trisaccharides (1% of total carbohydrates), being coincident with the fact that lactose was hydrolyzed faster than lactulose.

The presence of 3'-galactosyl-lactulose and β-Gal-(1→5)-Fru after 2h of reaction indicates that fructose and lactulose are acceptor substrates of galactose released during lactose hydrolysis.

The hydrolysis and transglycosylation occurs simultaneously at the same active site of glycosyl hydrolases (Abdul Manas et al., 2018). In consequence, the study of the transglycosylation mechanisms of BBMV glycosyl hydrolases, allows to know the mechanisms of hydrolysis of carbohydrates under physiological conditions through the identification and quantitation of yielded carbohydrates during the enzymatic reaction. In this work, the main trisaccharide linkage, using lactose, lactulose and a binary equimolecular mixture of these disaccharides, was β-(1→3). This result is aligned with the findings reported by Ferreira-Lazarte et al. (2019), who reported a higher hydrolysis rate of 3`-galactosyl-lactose contained in a commercial GOS mixture than 4′- and 6′-galactosyl-lactose following digestion by BBMV from pig small intestine. Also, the presence of reductive fructose in the oligosaccharide structure decreases the rate of synthesis and, possibly, its digestibility as well. This lower digestibility of novel GOS obtained from lactulose versus the conventional GOS obtained from lactose has been reported in studies that used *in vitro* (Ferreira-Lazarte et al., 2017) and *in vivo* digestibility models (Hernandez-Hernandez et al., 2012). This supports the hypothesis that the structures synthesized by the BBMV are more prone to be hydrolyzed and, therefore, to foresee their digestion fate under physiological conditions.

These data could be useful in the selection and design of potential low- or non-digestible prebiotic carbohydrates based on their structural characterization. However, the correlation between these data and *in vivo* studies must be conducted, as well as studies on other dietary and prebiotic carbohydrates, before firm conclusions can be drawn.

4. Conclusion

Although the glycosidase activity of BBMV towards lactulose is much lower than that observed for lactose, the synthesis of trisaccharides proceeds through the same mechanism for both disaccharides since preferably oligosaccharides linked by β-(1→3) (3'-galactosyl-lactulose and 3'-galactosyl-lactose, respectively) were synthesized. Fructose released during lactulose hydrolysis may be a suitable acceptor substrate for BBMV β-galactosidase since β-Gal-(1→5)-Fru was the main carbohydrate synthesized during lactulose transgalactosylation. The presence of free galactose in the reaction medium favors the formation of β-Gal-(1→5)-Fru and decreases that of 3'-galactosyl-lactulose. This study contributes to the understanding of the catalytic mechanism of mammalian small intestinal β−galactosidase and deepens in the knowledge of the correlation between the type of glycosidic bonds and carbohydrate digestibility.

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Conflict of interest

The authors declared no conflict of interest.

Appendix A.

Supplementary material

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Table 1. ^{1}H (500 MHz), ^{13}C (125 MHz) NMR chemical shifts[a] (δ, ppm) and coupling constants (J in Hz, in parentheses) of β-D-Galactopyranosyl–(1→3)-β-D-Galactopyranosyl-(1→4)-β-D-Fructose. (Fru: fructose; Gal: galactose).

		β-Pyranose		β-Furanose		α-Furanose	
	Position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
Fru	1	64.53	3.72, 3.56	63.12	3.56, 3.56	63.42	3.66, 3.66
	2	98.74	-----	103.01	-----	105.60	-----
	3	66.72	3.92	75.33	4.28	81.37	4.31
	4	78.02	4.14	84.76	4.26	85.90	4.09
	5	67.37	4.20	80.69	4.02	81.33	4.21
	6	63.62	4.01, 3.74	63.29	3.80, 3.71	63.09	3.79, 3.69
Gal-2	1	101.10	4.615 (7.8)	103.16	4.51 (≈8)	103.59	4.49 (≈8)
	2	70.59	3.77	70.58	3.72	70.58	3.73
	3	82.77	3.83	82.61	3.82	82.55	3.82
	4	69.13	4.19	68.96	4.18	68.96	4.18
	5	75.66	3.72	75.57	3.72	75.57	3.72
	6	61.75	3.81, 3.75	61.64	3.78, 3.74	61.64	3.78, 3.74
Gal-1	1	104.98	4.610 (7.8)	104.98	4.610 (7.8)	104.98	4.610 (7.8)
	2	71.68	3.61	71.68	3.61	71.68	3.61
	3	73.16	3.65	73.16	3.65	73.16	3.65
	4	69.22	3.91	69.22	3.91	69.22	3.91
	5	75.72	3.68	75.72	3.68	75.72	3.68
	6	61.61	3.75, 3.75	61.61	3.75, 3.75	61.61	3.75, 3.75

Figure captions

Figure1.- GC-FID chromatographic profiles of TMSO derivatives of: **(a)** transgalactosylated lactulose, **(a_1)**: disaccharide fraction, **(a_2)**: trisaccharide fraction and **(b)** transgalactosylated lactose/lactulose 50/50 mixture, **(b_1)**: disaccharide fraction **(b_2)**: trisaccharide fraction.

Figure 2. Assignment of ^{13}C NMR spectrum of β-D-galactopyranosyl - (1-3) -β-D-galactopyranosyl- (1-4) -D-fructose in D_2O. □ β-D-galactopyranosyl - (1-3) -β-D-galactopyranosyl- (1-4) -β-D-fructopyranoside. ⬭ β-D-galactopyranosyl - (1-3) -β-D-galactopyranosyl- (1-4) -β-D-fructofuranoside. ▲ β-D-galactopyranosyl - (1-3) -β-D-galactopyranosyl- (1-4) -α-D-fructofuranoside.

Figure 3.- Tautomeric forms of the β-D-galactopyranosyl - (1-3) -β-D-galactopyranosyl- (1-4) -D-fructose characterized by NMR.

Figure 4.- Hydrolysis **(a)** and transgalactosylation **(b)** reactions of lactulose catalyzed by β-galactosidase from pig small intestinal BBMV. ◇fructose, ▲ galactose, □ lactulose, ♦ β-Gal-(1→4)-Gal (1,4 galactobiose), ✳ β-Gal-(1→5)-Fru and △ β-Gal-(1-3)-β-Gal-(1-4)-β-Fru.

Figure 5.- Hydrolysis **(a)** and transgalactosylation **(b)** reactions of 80/20 lactulose/galactose mixtures catalyzed by β-galactosidase pig small intestinal BBMV. ◇ fructose, ▲ galactose, □ lactulose, ♦ β-Gal-(1→4)-Gal (1,4 galactobiose), ✳ β-Gal-(1→5)-Fru and β-Gal-(1→3)-β-Gal-(1→4)-β-Fru.

Figure 6.- Hydrolysis **(a)** and transgalactosylation **(b)** reactions of 50/50 lactulose/lactose mixture catalyzed by β−galactosidase from pig small intestinal BBMV. ◇ fructose, ▲ galactose, ● glucose, □ lactulose, ■ lactose, ♦ β-Gal-(1→4)-Gal (1,4 galactobiose), ✳ β-Gal-(1→5)-Fru, ○ β-Gal-(1 → 2)-Glc, △ β-Gal-(1 →3)-Glc, ● β-Gal-(1→6)-Glc (allolactose), ◇ β-Gal-(1 → 3)-β-Gal-(1 → 4)-Glc, △ β-Gal-(1→3)-β-Gal-(1→4)-β-Fru and ■ β-Gal-(1→4)-β-Glc-(1↔1)-β-Gal and β-Gal-(1→3)-Glc-(2→1)-β-Gal mixture.

Figure 1

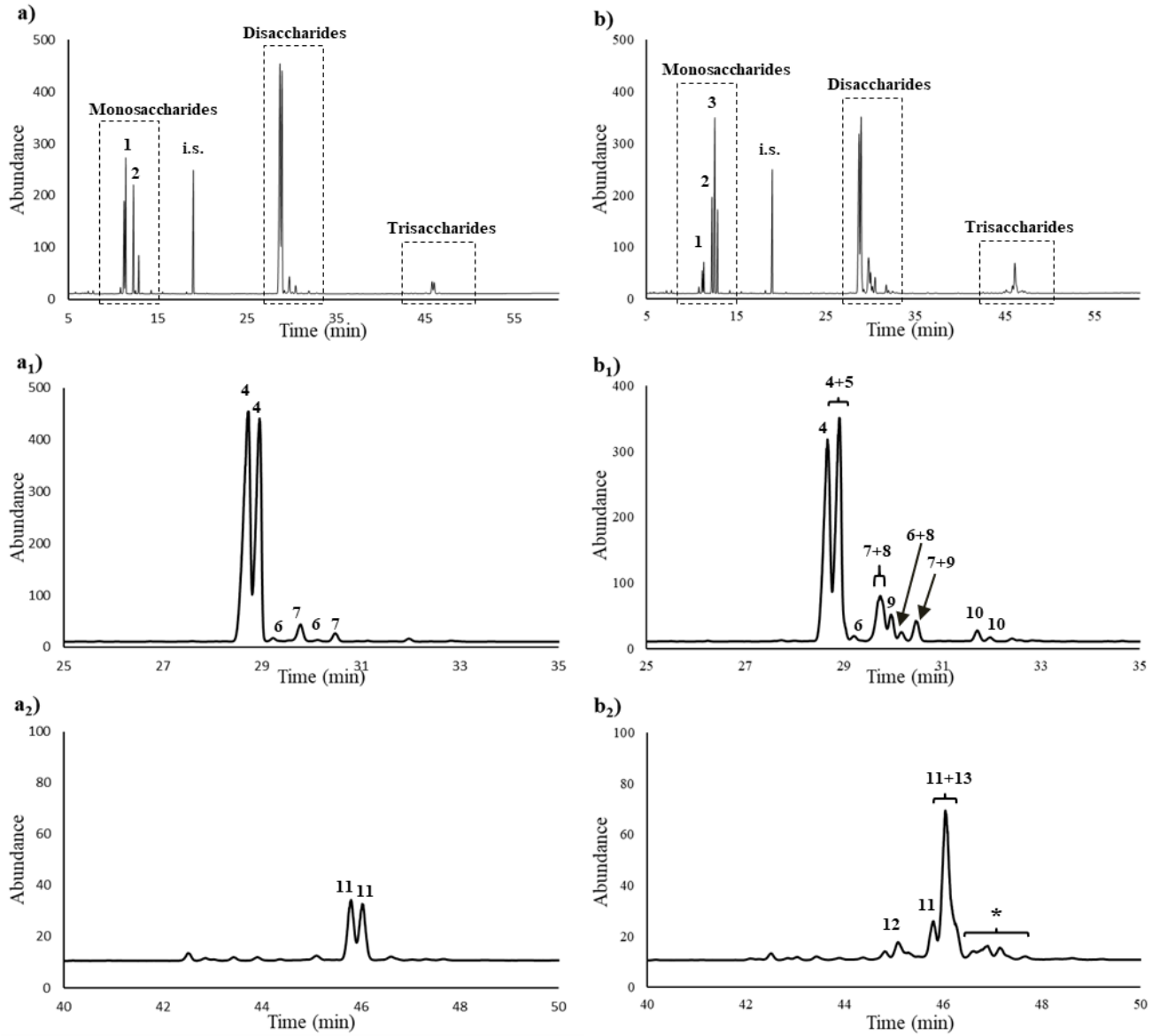

a)
Monosaccharides
Disaccharides
Trisaccharides
i.s.
1
2
Abundance
Time (min)
b)
Monosaccharides
Disaccharides
Trisaccharides
i.s.
1
2
3
Abundance
Time (min)
a1)
4
4
6
7
6
7
Abundance
Time (min)
b1)
4+5
4
6
7+8
6+8
7+9
9
10
10
Abundance
Time (min)
a2)
11
11
Abundance
Time (min)
b2)
11+13
12
11
*
Abundance
Time (min)

Figure 2

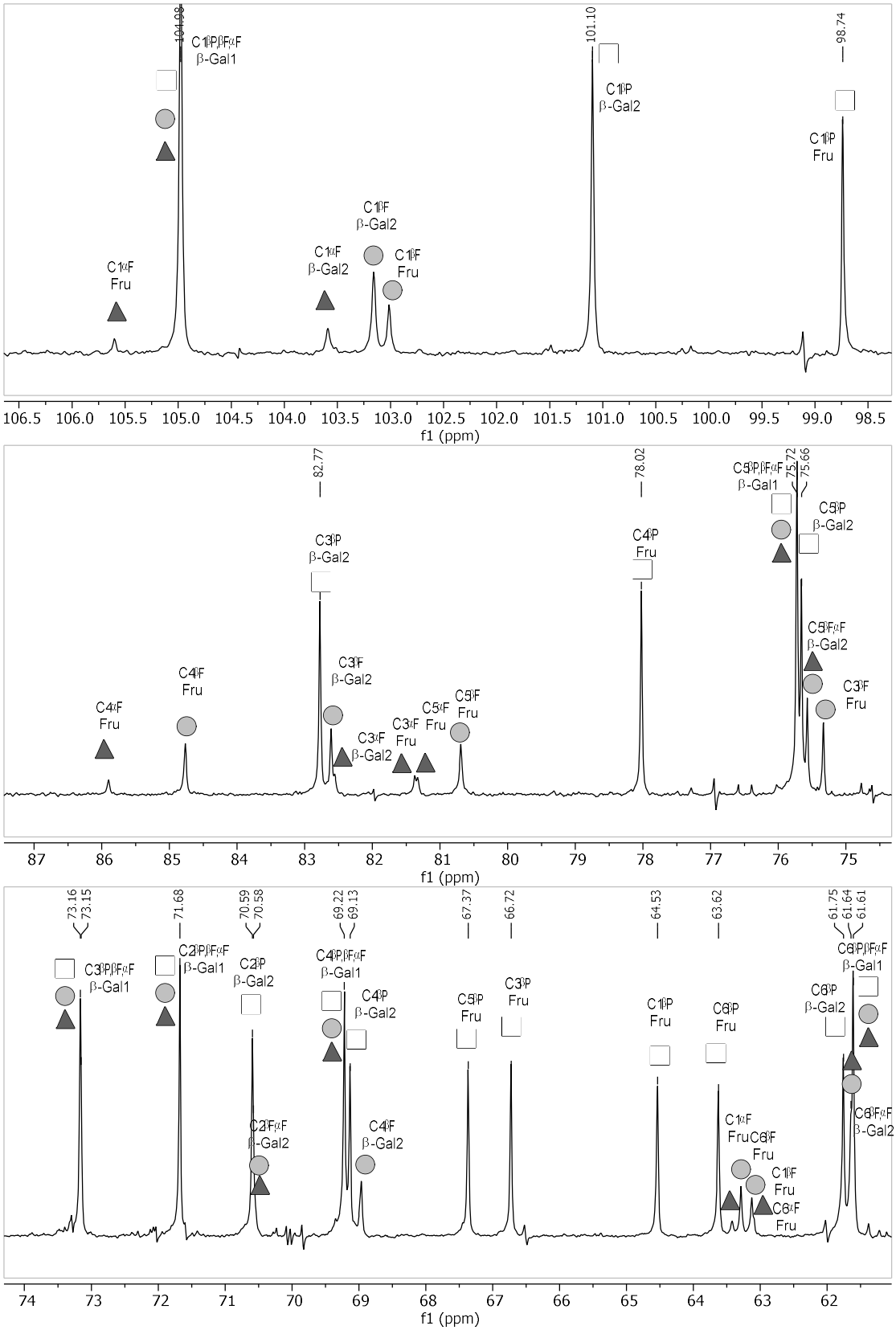
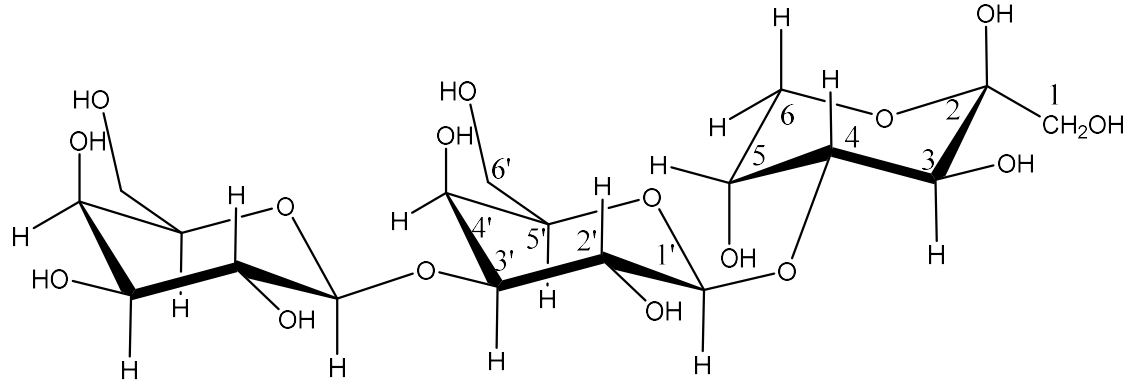
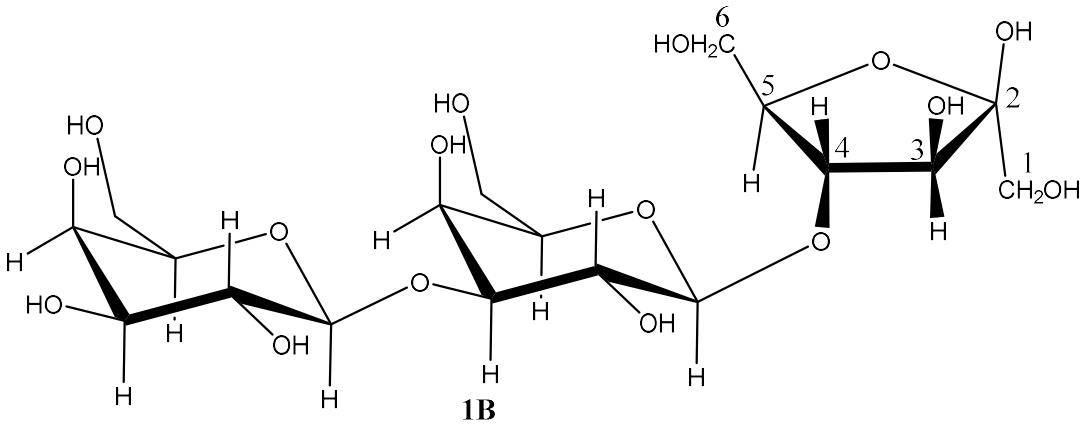

104.98
C1βP,βF,αF
β-Gal1
101.10
C1βP
β-Gal2
98.74
C1βP
Fru
C1βF
β-Gal2
C1αF
β-Gal2
C1βF
Fru
C1αF
Fru
106.5 106.0 105.5 105.0 104.5 104.0 103.5 103.0 102.5 102.0 101.5 101.0 100.5 100.0 99.5 99.0 98.5
f1 (ppm)
82.77
78.02
75.72
75.66
C5βP,βF,αF
β-Gal1
C5βP
β-Gal2
C4βP
Fru
C3βP
β-Gal2
C5βF,αF
β-Gal2
C3βF
β-Gal2
C4βF
Fru
C4αF
Fru
C3αF
Fru
C5αF
Fru
C5βF
Fru
C3αF
β-Gal2
C3βF
Fru
87 86 85 84 83 82 81 80 79 78 77 76 75
f1 (ppm)
73.16
73.15
71.68
70.59
70.58
69.22
69.13
67.37
66.72
64.53
63.62
61.75
61.64
61.61
C3βP,βF,αF
β-Gal1
C2βP,βF,αF
β-Gal1
C2βP
β-Gal2
C4βP,βF,αF
β-Gal1
C4βP
β-Gal2
C5βP
Fru
C3βP
Fru
C1βP
Fru
C6βP
Fru
C6βP
β-Gal2
C6βP,βF,αF
β-Gal1
C2βF,αF
β-Gal2
C4βF
β-Gal2
C1αF
Fru
C6βF
Fru
C1βF
Fru
C6αF
Fru
C6βF,αF
β-Gal2
74 73 72 71 70 69 68 67 66 65 64 63 62
f1 (ppm)

Figure 3



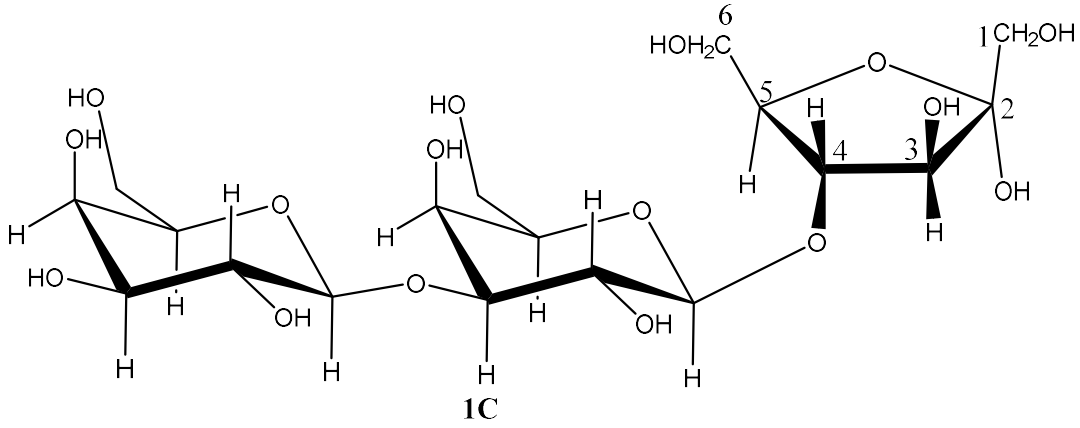
1A

β-D-Galactopyranosyl - (1-3) -β-D-Galactopyranosyl- (1-4) -β-D-Fructopyranoside



1B

β-D-Galactopyranosyl - (1-3) -β-D-Galactopyranosyl- (1-4) -β-D-Fructofuranoside



1C

β-D-Galactopyranosyl - (1-3) -β-D-Galactopyranosyl- (1-4) -α-D-Fructofuranoside

Figure 4

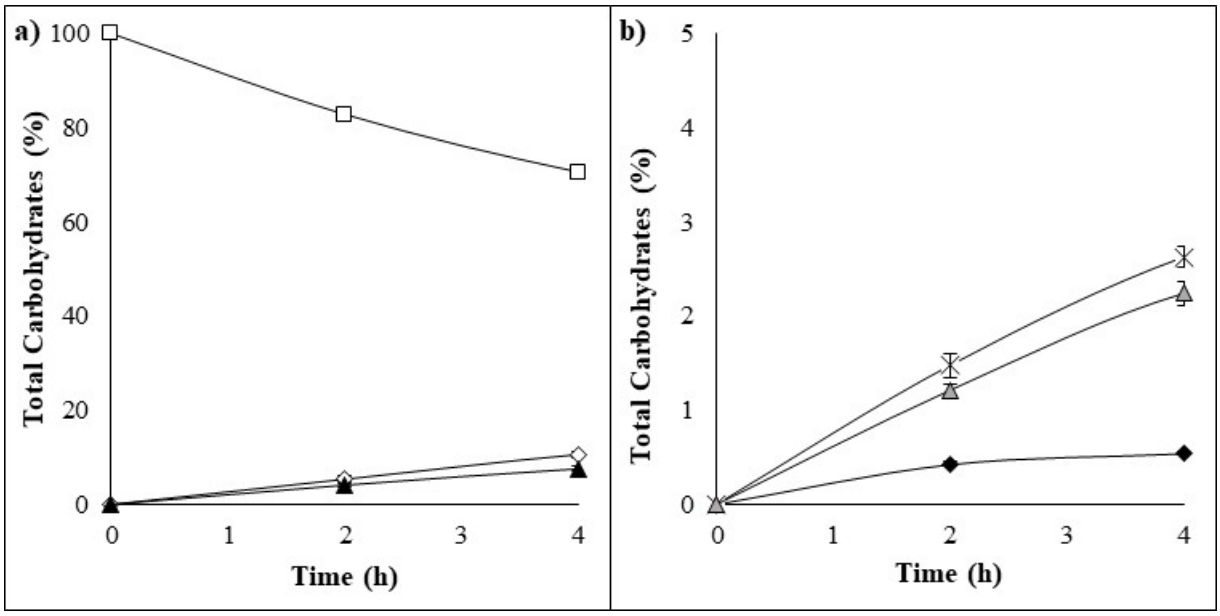

a)
Total Carbohydrates (%)
100
80
60
40
20
0
0
1
2
3
4
Time (h)
b)
Total Carbohydrates (%)
5
4
3
2
1
0
0
1
2
3
4
Time (h)

Figure 5

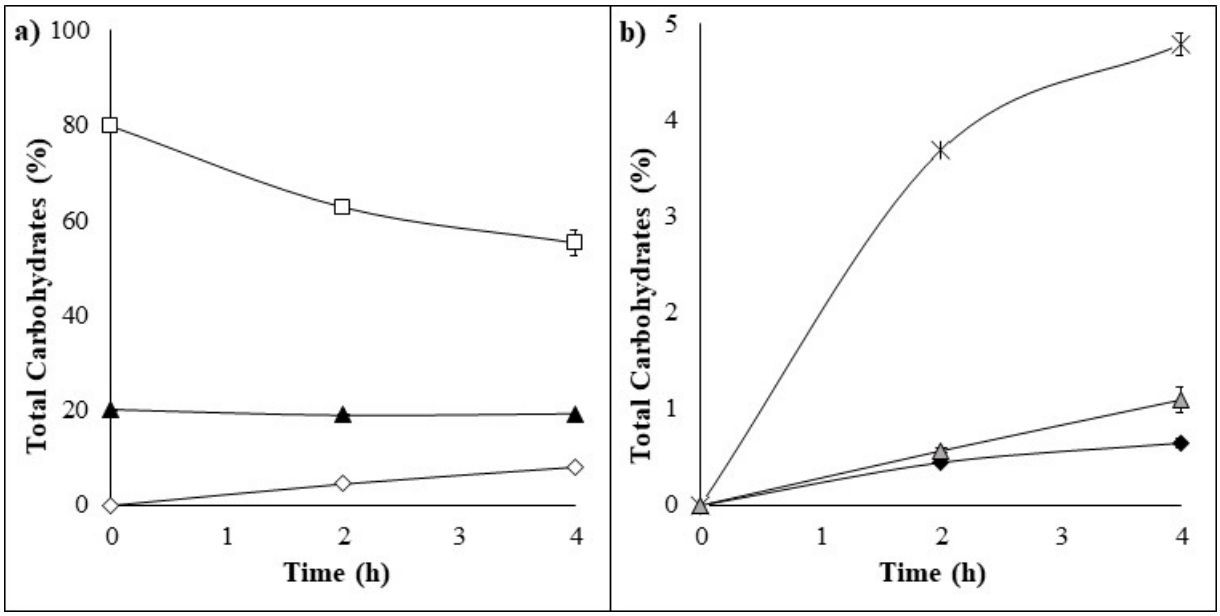
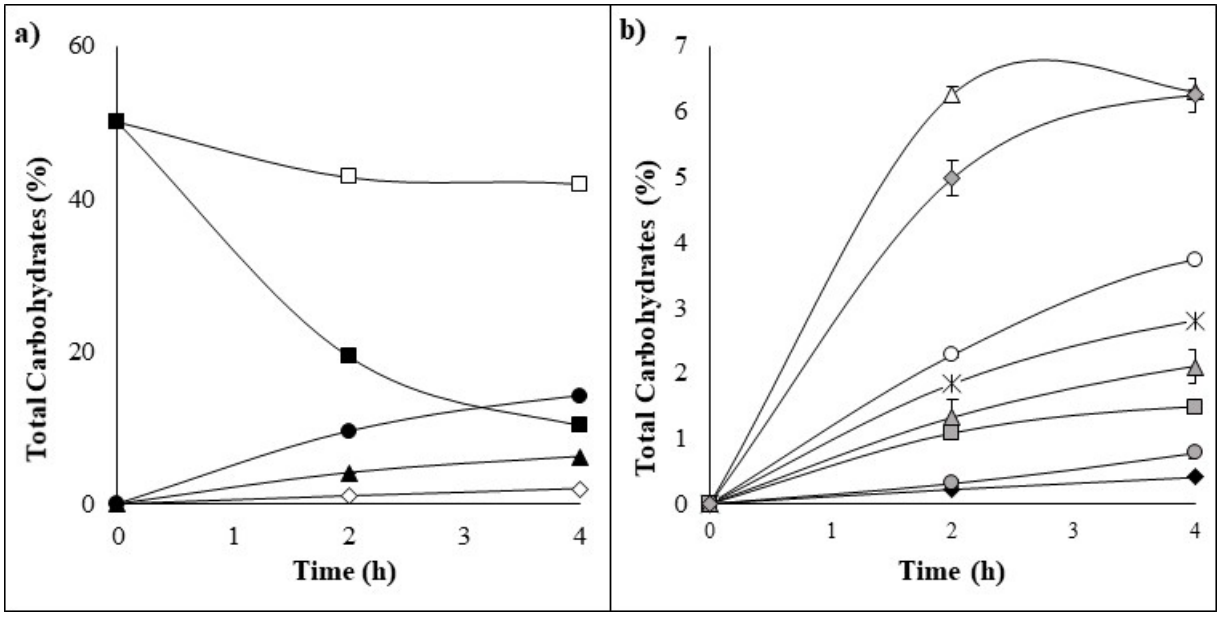

a)
Total Carbohydrates (%)
100
80
60
40
20
0
0
1
2
3
4
Time (h)
b)
Total Carbohydrates (%)
5
4
3
2
1
0
0
1
2
3
4
Time (h)

Figure 6


a)
Total Carbohydrates (%)
60
40
20
0
0
1
2
3
4
Time (h)
b)
Total Carbohydrates (%)
7
6
5
4
3
2
1
0
0
1
2
3
4
Time (h)

Supporting Information

Hydrolysis and transgalactosylation catalysed by β-galactosidase from brush border membrane vesicles isolated from pig small intestine: A study involving different substrates

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Table of Contents

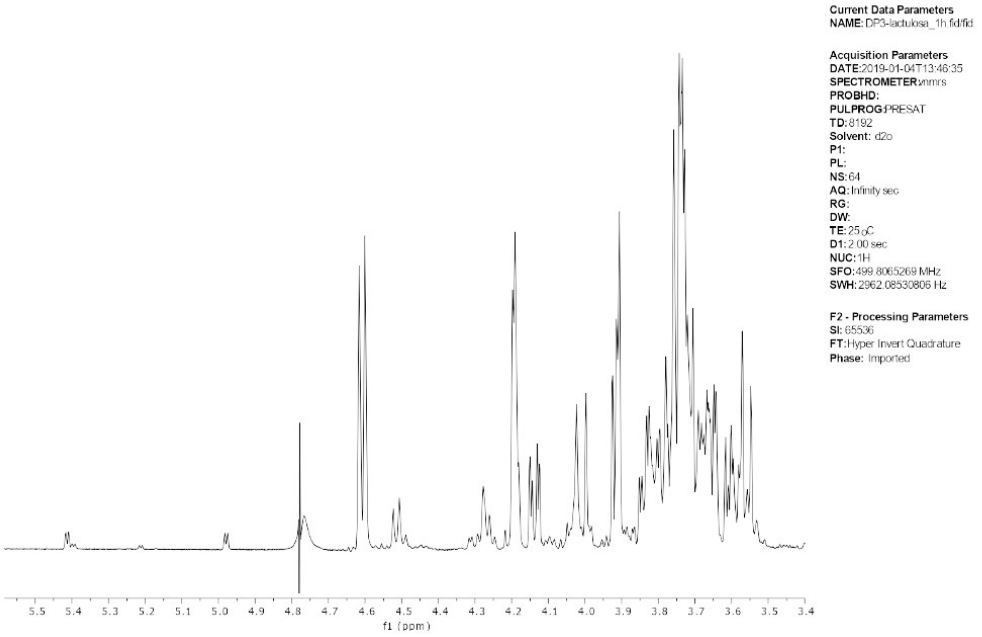

Figure S1. ^{1}H NMR (500 MHz, D2O) of β-D-Galactopyranosyl – (1→3) -β-D-Galactopyranosyl- (1→4) -β-D-fructose.

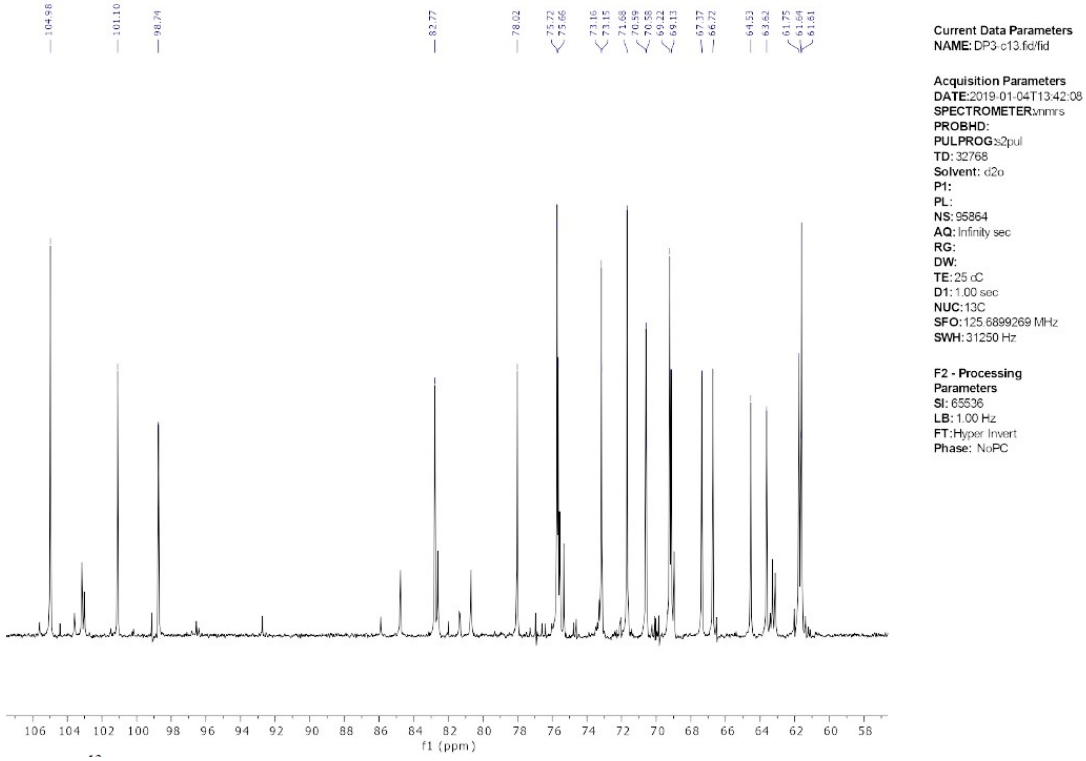

Figure S2. ^{13}C NMR (125 MHz, D2O) of β-D-Galactopyranosyl – (1→3) -β-D-Galactopyranosyl- (1→4) -β-D-fructose.

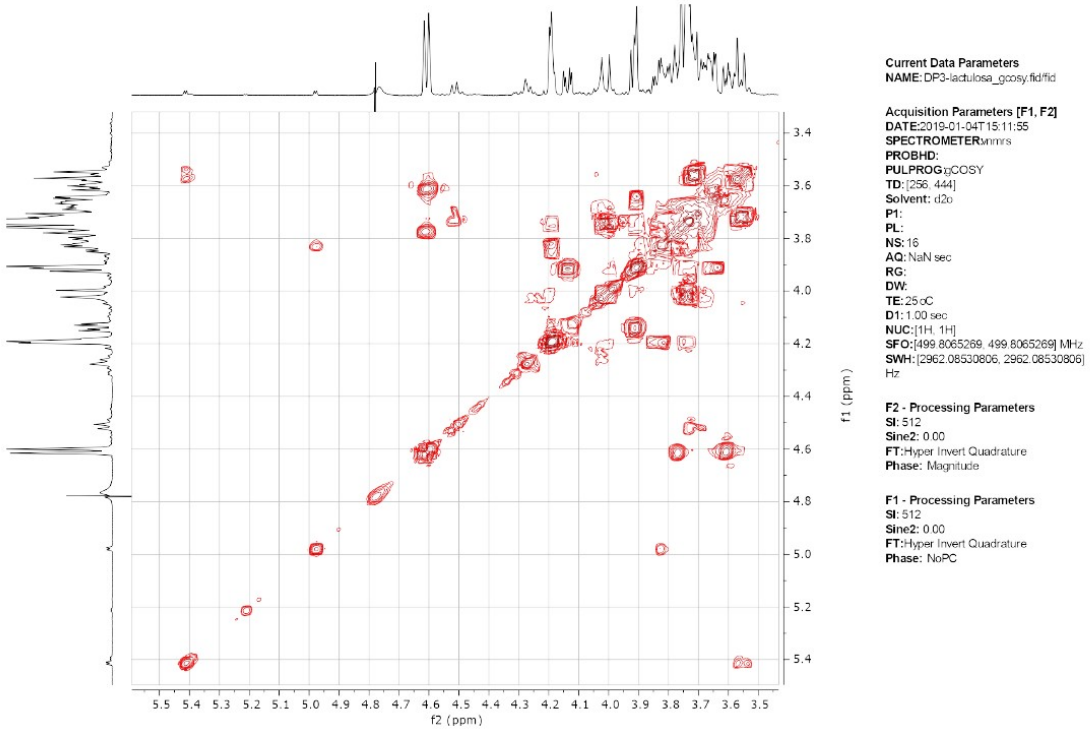

Figure S3. gCOSY (500 MHz, D2O) of β-D-Galactopyranosyl – (1→3) -β-D-Galactopyranosyl- (1→4) -β-D-fructose.

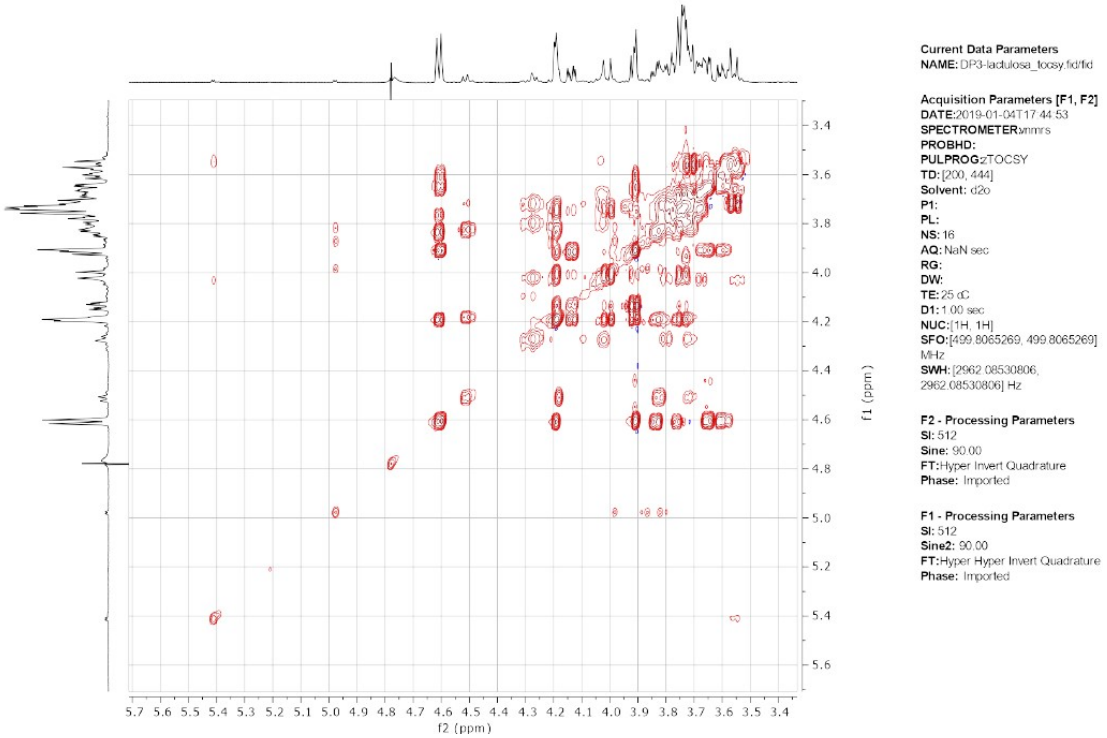

Figure S4. TOCSY (500 MHz, D2O) of β-D-Galactopyranosyl – (1→3) -β-D-Galactopyranosyl- (1→4) -β-D-fructose.

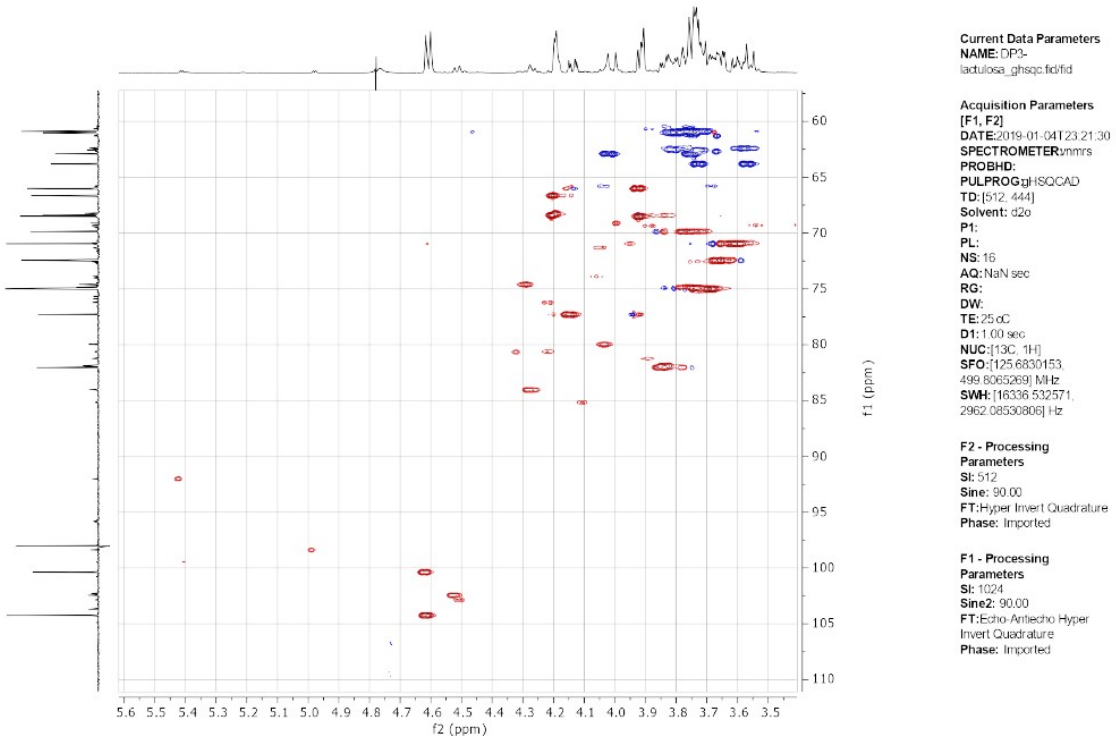

Figure S5. Multiplicity-edited gHSQC (500 MHz, D2O) of β-D-Galactopyranosyl – (1→3) -β-D-Galactopyranosyl- (1→4) -β-D-fructose. **(**methylene**:** blue cross peaks; methine**:** red cross peaks)

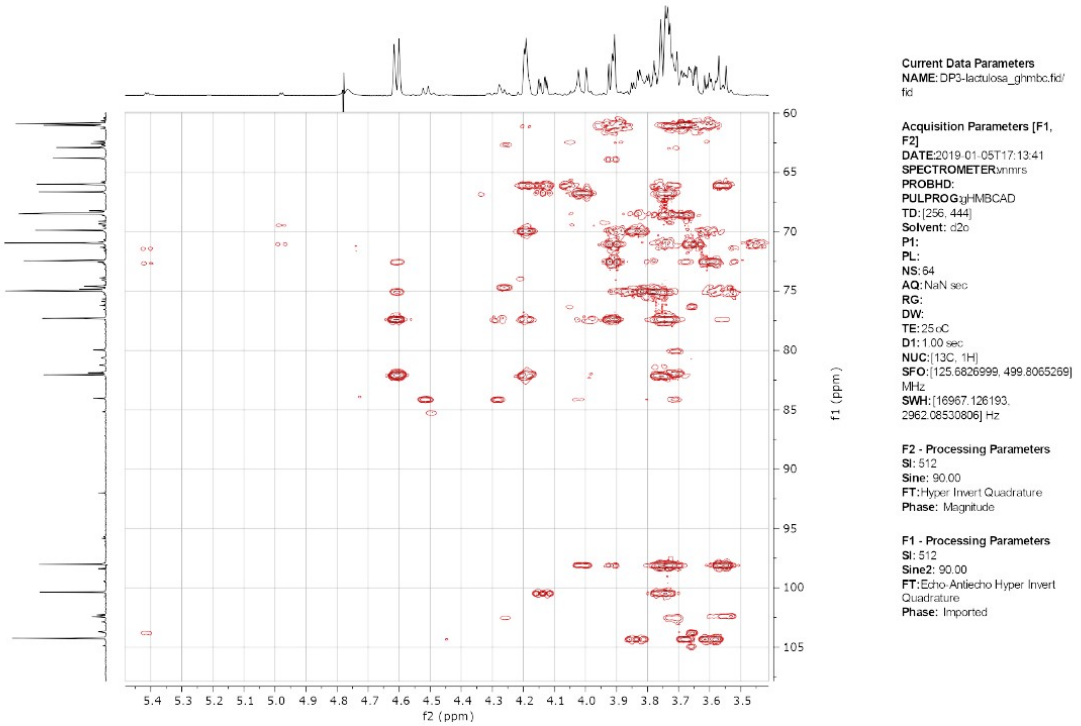

Figure S6. gHMBC (500 MHz, D2O) of β-D-Galactopyranosyl – (1→3) -β-D-Galactopyranosyl- (1→4) -β-D-fructose.

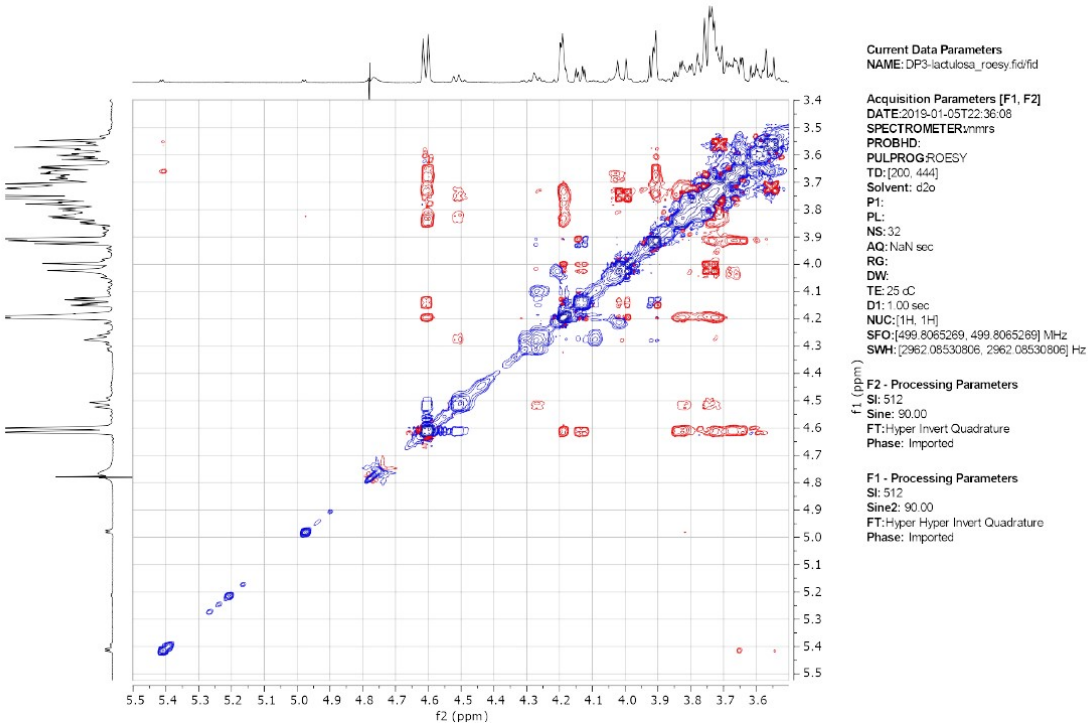

Figure S7. ROESY (500 MHz, D2O) of β-D-Galactopyranosyl – (1→3) -β-D-Galactopyranosyl- (1→4) -β-D-fructose.